AD_____

Award Number: DAMD17-97-1-7230

TITLE: Cell Adhesion, Signaling and Myosin in Breast Cancer

PRINCIPAL INVESTIGATOR: Luraynne Sanders, Ph.D.

CONTRACTING ORGANIZATION: The Scirpps Research Institute
La Jolla, California 92037

REPORT DATE: August 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Annual Summary (1 Aug 99 - 31 Jul 00)

4. TITLE AND SUBTITLE
Cell Adhesion, Signaling and Myosin in Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7230

6. AUTHOR(S)
Luraynne Sanders, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Scirpps Research Institute
La Jolla, California 92037

E-MAIL:
sanders@scripps.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Cytoskeleton remodeling is crucial in many cellular events, including cell adhesion, spreading and motility. The Rho family of small GTPases (Rho, Rac and Cdc42) are signal transducers that regulate cytoskeleton dynamics. However, little is known about the mechanisms by which Rho GTPases induce cytoskeletal changes. Here we show that Rac1 is important for cell spreading, a biological process in which the cytoskeleton is highly active. Overexpression of dominant negative or constitutively active Rac1 significantly impairs the ability of the cells to spread. Rac activity is high only during early stages of spreading. The activity of p-21activating kinase (PAK), an effector molecule for Rac and Cdc42, is also high only during the early stages of spreading. Overexpression of catalytically active PAK inhibits cell spreading and decreases myosin phosphorylation. Myosin is the cytoskeleton protein which provides the force generating ability to the actin cytoskeleton. Treatment of cells with myosin inhibitors also inhibits cell spreading. In vitro and in vivo studies revealed a novel target for PAK, myosin light chain kinase (MLCK), the enzyme that phosphorylates the light chain of myosin II. Activated PAK phosphorylates MLCK and inhibits its ability to phosphorylate MLC. Thus, PAK appears to modulate cytoskeleton changes by phosphorylation and downregulation of MLCK.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	13
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

YS Where copyrighted material is quoted, permission has been obtained to use such material.

YS Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

YS Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

YS For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

YS In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

YS In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

YS In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Suzanne Sanders 10/24/00

PI - Signature Date

Table of Contents

Introduction

Tumor invasion and metastasis are the major cause of cancer mortality. This is particularly true for breast cancer since metastasis of the primary tumor leads to tumor inaccessibility and, consequently, greater mortality. Many studies have shown the importance of signaling molecules in changes that are associated with transformed phenotypes (1-3). In fact, many signaling molecules are affiliated with cytoskeletal changes that accompany the motile or metastatic phenotype. The Rho family of small GTPases (Rho, Rac and Cdc42) are members of the Ras superfamily; these regulate cell function via conversion between a GTP-bound active state and a GDP-bound inactive form. Recently, it has become clear that the Rho family mediates morphological and cytoskeletal changes of both normal and transformed cells (4,5). Rho activation leads to stress fiber formation and focal adhesions. Activation of Rac leads to membrane ruffles and lamellipodia formation. Similarly, Cdc42 regulates the extension of actin filament bundles into filopodia. The molecular mechanisms by which the Rho family of GTPases regulates cytoskeleton remodeling is not well understood.

Dynamic rearrangement of the cytoskeleton is, in part, driven by actin polymerization/depolymeriation and actin-myosin II interactions. Myosins II (which are found in all cells and are constructed of two heavy chains and four light chains) are mechanoenzymes which generate force along actin filaments and, thus, are crucial for cell movements, including cytokinesis, pseudopod formation, polarized growth and cell migration (6-8). Changes in the expression of myosin isoforms have been linked to the transformed phenotype in both melanoma and breast cancers (9-11). Recently, Rho GTPase has been shown to regulate myosin activity though Rho kinase, an effector molecule for Rho (12,13). Rho kinase phosphorylates myosin phosphatase, which removes phosphate groups from myosin light chain, and inhibits its function. Consequently, one mechanism by which Rho effects cytoskeletal dynamics is by increasing myosin light chain phosphorylation through the inhibition of dephosohorylation.

In this grant we proposed to examine the effects p21-activated kinase (PAK), an effector molecule for Rac and Cdc42, had on cytoskeleton dynamics and how this relates to the phenotype and behavior of breast cancer cells.

Results

Myosin light chain kinase (MLCK) increases in phosphorylation at Ser-19 on myosin light chain (MLC) are essential for force generation by myosin II. During postmitotic cell spreading, phosphorylation of this site is elevated when compared to completely spread cells (14). The spreading edge of a cell is analogous to the leading edge of a migrating cell and collectively these are referred to as moving edges. Furthermore, cell spreading is a prerequisite for cell migration. Aim 2 of this grant was to evaluate the effect PAK may have on myosin II phosphorylation. Therefore, we looked at the effects PAK had on MLC phosphorylation during cell spreading. When BHK-21 cells were transfected with PAK1 T423E (catalytically active) and placed in an adhesion assay, spreading was largely inhibited. However, cells expressing Lac Z or PAK WT spread normally (15). Inhibitors of myosin or MLCK showed similar results to PAK T423E expressing cells. Cells overexpressing PAK T423E, PAK WT, Lac Z, or control cells (non-transfected) were allowed to attach and spread on a fibronectin matrix, then lysed at various time points. Immunoblot analysis was performed using an antibody that recognizes the Ser-19 phosphorylated form of MLC (18). During cell spreading the control cells show a gradual increase in MLC phosphorylation, with the maximum at the 45-min time point. However, PAK1 T423E expressing cells show little to no phosphorylation of MLC at any of the time points. Cells transfected with Lac Z or PAK WT showed a level of MLC phosphorylation similar to control cells. These data suggest that in vivo, catalytically active PAK1 acts to inhibit phosphorylation of MLC on Ser-19 (15).

The calcium-calmodulin dependent myosin light chain kinase (MLCK) phosphorylates MLC on Ser-19 and is known in vivo to be responsible for promoting the force generating ability of myosin II. Therefore, in order to understand PAK's role in decreasing phosphorylation of MLC, we looked at its effect on MLCK. In vitro phosphorylation experiments demonstrate that PAK 1 can phosphorylate MLCK and this phosphorylation is independent of calmodulin. PAK 1 phosphorylation of MLCK lead to a 50% reduction in the catalytic activity of MLCK (15). This data suggests that catalytically active PAK inhibits MLC phosphorylation by phosphorylating MLCK and downregulating its activity.

To test the ability of PAK to inactivate MLCK in vivo, cells were transfected with PAK1 WT and T423E, lysed, and MLCK was immunopercipitated and assayed for activity. MLCK immunopercipitated from PAK T423E cells showed a significant decrease in activity when compared to MLCK from control or PAK WT expressing cells (15). This data confirms our in vitro data that PAK phosphorylation of MLCK inhibits its activity.

We have also found that PAK phosphorylates and activates another enzyme important in controlling cytoskeleton dynamics, Lim-kinase (16). Lim-kinase phosphorylates and inactivates the small actin binding protein cofilin/actin depolymerizing factor (ADF). Recent studies have indicated Lim-kinase acts downstream of Rac (17). PAK phosphorylated Lim-kinase at threonine 508 and increased Lim-kinase-mediated phosphorylation of cofilin ~10 fold in vitro. In vivo, activated Rac increased association

of PAK with Lim-kinase, which required structural determinants in both the NH_2-terminal regulatory and COOH-terminal catalytic domains of PAK. These data suggest a model by which the activation of PAK by Rac/Cdc42 leads to enhanced binding of Lim-kinase and effective phosphorylation by PAK. A catalytically inactive Lim-kinase interfered with Rac-, Cdc42-and PAK-dependent cytoskeletal changes. A PAK-specific inhibitor, corresponding to the PAK autoinhibitory domain, blocked Lim-kinase-induced cytoskeletal changes.

Aim 3 of this grant was to examine the functional importance of PAK on breast cancer cell migration. Detailed cellular analysis of breast cancer cell lines has been hindered by the low protein expression and number of transfected cells that microinjection and other traditional methods render. Therefore, we perfected a viral gene expression system to overcome this problem. This system has allowed us to transiently express Rho family GTPases and PAKs with an efficiency of greater than 95% in many breast cancer cell lines. We are now able to study a population of cells that can be placed into bioassays, including migration or adhesion assays. Using the virus system to transfect cells, our preliminary results indicate that high levels of activated PAK decreases cell migration. This would be consistent with PAK's ability to decrease MLCK activity and increase Lim-kinase activity.

Recently, in collaboration with the Knaus lab here at Scripps, we have shown that some breast cancer cell lines have constitutively activated PAK (18). Most of the cell lines showing increased PAK activity also had constitutively active Rho GTPase. However, two of these cell lines, ZR-75 and SK-BR-3, have activated PAK with no activated GTPase. These two cell lines have abnormal cytoskeletal morphologies and are very poor migraters in both boyden chamber or woudhealing assays. The actin filaments are thicker and focal adhesions are much larger when compared to breast cancer cell lines which do not have endogenous activated PAK (i.e. MDA-231 cells, data not shown) or normal fibroblast cells (i.e. BHK cells) (figure 1). The thicker actin filaments and adnormaly large focal adhdsion would explain why both the SK-BR-3 and ZR-75 cells migrate poorly. The thicker actin filaments in SK-BR-3 and ZR-75 cells is reminiscent of the morphology observed in cells overexpressing Lim-kinase (16); and indeed cell lysates from these cells immunoblotted with a antibody that detects the phosphoylated form of cofilin show increased cofilin phosphorylation when compared to cell lines without elevated PAK activity (figure 2). Furthermore, when SK-BR-3 or ZR-75 cells are immunostained with an antibody that recognizes the active form of PAK the immunoreactivity is localized to the focal adhesions (figure3), this antibody does not stain focal adhesions of BHK (fibroblast) or MDA 231 cells (breast cancer cell line without endogenous active PAK). These data suggest that PAK is inappropriately recruited to focal adhesions in the breast cancer cells with high PAK activity. Inhibition of PAK, by overexpression of the PAK of the PAK autoinhibitory domain, leads to a reduction in the actin filaments (figure 4).

Conclusion

Cell adhesion, migration and invasion play critical roles in the pathogenesis of tumor metastasis. Comprehending cytoskeleton dynamics is pivotal in understanding the complexities of metastasis. Thus, our studies have first focused on cell spreading in order to understand the complexities of the cytoskeleton at the moving edge of the cell. Our lab and others have shown that PAK localizes to Rac-induced membrane ruffles (20) and mutationally active forms can cause cytoskeleton changes (19, 20, 21). These data suggest a role for PAK in cytoskeletal remodeling. Data presented in this report further demonstrate the importance of PAK and suggest a possible mechanism by which it influences the cytoskeleton. We describe two novel targets for PAK, MLCK and Lim-kinase. Both of these kinases are important regulators of cytoskeleton dynamics. Aim 2 in our grant proposal was to determine if PAK affected myosin phosphorylation (Technical Objective 2: task 3 and 4). We believe that these results satisfy the objective of Aim 2.

At present we are working on Aim 3 in our proposal, which is to examine the importance of PAK-myosin interactions on breast cancer cell migration. With the development of the viral gene expression system, we are now able to transfect breast cancer cells with high enough transfection efficiency to do migration (Technical Objective 3: task 5 and 6). These experiments are currently ongoing.

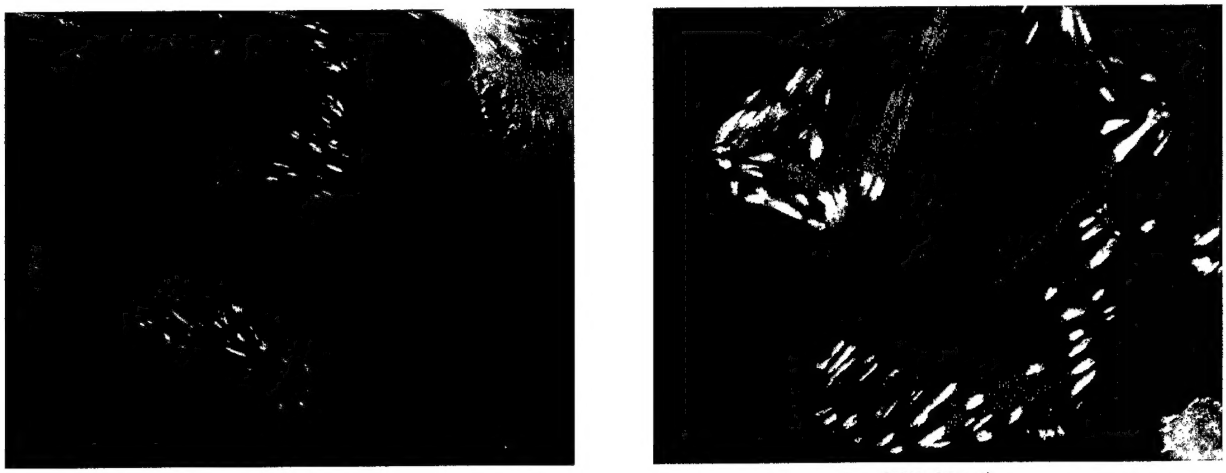


Figure 1: Breast Cancer Cells with Activated PAK have Larger Actin Filaments and Focal Adhesions. BHK and SK-BR-3 cells were stained with phalloidin (red) to reveal the actin cytoskeleton and anti-hVinculin (green) to visualize the focal adhesions. Focal adhesions are structures within the cell which contain molecules essential for the cell to sense its environment. Actin filaments end within these structures, which are crucial for cell migration. Where the actin filaments and focal adhesions meet the two dyes fluoresce yellow. **A)** BHK cells. **B)** SK-BR-3 cells, breast cancer cells with high PAK activity, have much larger focal adhesions and actin filaments then the normal BHK cells.

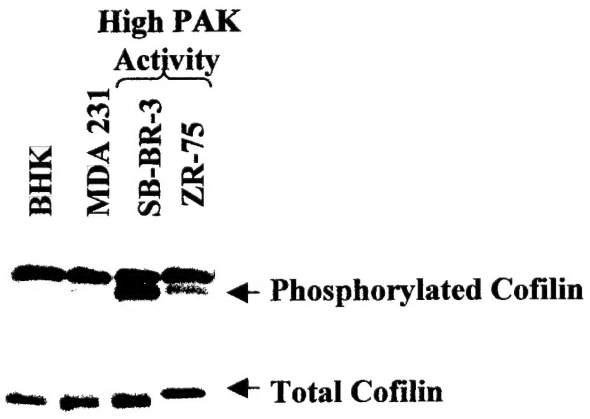
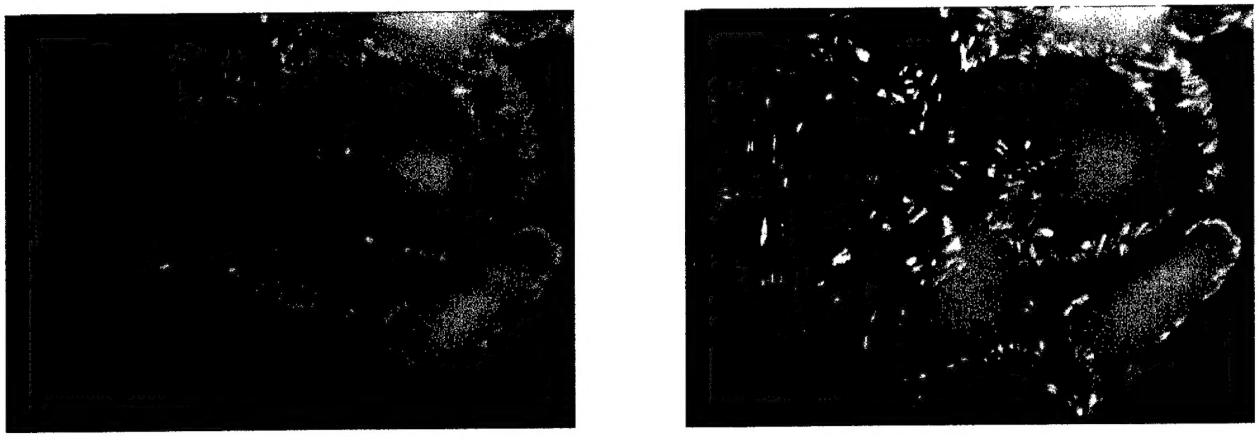


Figure 2: Breast Cancer Cells with High PAK Activity have Increased Cofilin Phosphorylation. In several breast cancer cell lines PAK activity is upregulated (18). Breast cancer cells with endogenous activated PAK have increased phosphorylated cofilin, consistent with the observed thickening of actin filaments seen in cells overexpressing Lim-kinase (16). Cell lysates from SB-BR-3 and ZR-75 show increased cofilin phosphorylation when compared to cell lines without elevated PAK activity. The lower panel shows comperable levels of cofilin in the lysates.



Anti-pPAK

Merge: Anti-pPAK and Anti-hVinculin

Figure 3: Breast Cancer Cells with High PAK Activity Mislocalize PAK to Focal Adhesions. SK-BR-3 cells were stained with an antibody that recognizes activated PAK (Anti-pPAK in green), and co-stained with anti-hVinculin (red) to visualize focal adhesions.The merge (orange) demonstrates active PAK localizes to these structures. ZR-75 cells also stain for active PAK in focal adhesions; however, BHK and MDA-231 cells (cells without elevated PAK activity) do not show localization of PAK to focal adhesions. These data suggest that PAK is inappropriately recruited to focal adhesions in the breast cancer cells with high PAK activity.

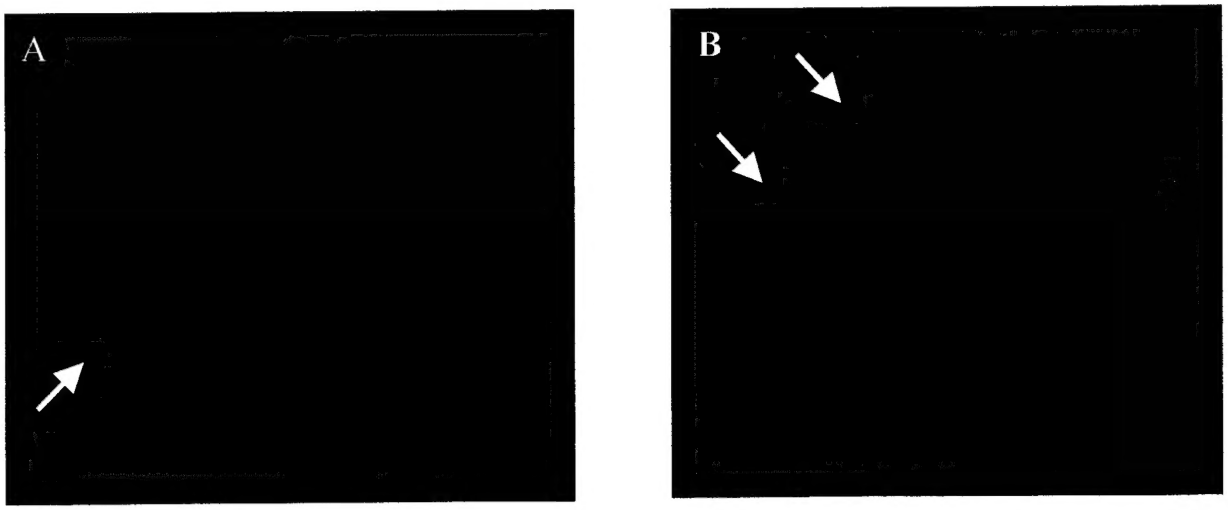


Figure 4: Inhibition of PAK Reduces Actin Filaments in Breast Cancer Cells. Inhibition of PAK in SK-BR-3 cells, by overexpression of the PAK autoinhibitory domain, leads to a reduction in actin filaments. **A)** SK-BR-3 cell overexpressing the autoinhibitory domain of PAK, arrow indicates transfected cells. **B)** SK-BR-3 cells overexpressing a control protein (LacZ) show no change in cytoskeletal morphology, arrows indicate transfected cells.

References

1. Qui, R-G., J. Chen, D. Kirn, F. McCormick and M. Symons (1995) An essential role for rac and ras transformation. *Nature*, 374:457-459

2. Prendergast, G.C., R. Khosravi-Far, P.A. Solski, H. Kurzowa, P.F. Lebowitz and C.J. Der (1995) Critical role of rho in cell transformation by oncogenic ras *Oncogene*, 10:2289-2296

3. Renshaw, M.W., E. Lea-Chou, G.M. Bokoch and J.P. Wang (1995) Rac is required for v-abl tyrosine kinase to activate mitogenesis. *Nature*, submitted

4. Ridley, A.J., H. F. Paterson, C.L. Johnston, D. Diekmann and A. Hall (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*, 70:401-410

5. Hall, A. (1994) Small GTP-binding proteins and the regulation of the actin cytoskeleton. Ann. Rev. *Cell Biol.*, 10: 31-54

6. Wolenski, J.S. (1995) Regulation of calmodulin-binding myosins. *Trends Cell Bio* 5:310- 316.

7. de Lanerolle, P. and Paul, R.J. (1991) Myosin phosphorylation/dephosphorylation and regulation of airway smooth muscle contractility. *Am. J. Physiol.*, 261:L1-L14.

8. Wilson, A.K., Pollenz, R.S., Chisholm, R.L. and de Lanerolle, P. (1992) The role of myosin I and II in cell motility. *Cancer and Metastasis Reviews*, 11:79-91.

9. Kelley, C.A. and R.S. Adelstein (1990) The 204-kDa smooth muscle myosin heavy chain is phosphorylated in intact cells by casein kinase II on a serine near the carboxyl terminus. *J. Bio. Chem.*, 265:17876-17882

10. Fink-Puches, R., and J. Smolle (1993) Cytoskeleton and motility: An immunohistological and computer simulation analysis of melanocytic skin tumors. *J. Cutan. Path.*, 20:130-136

11. Maupin, P., C.L. Phillips, R.S. Adelstein and T.D. Pollard (1994) Differential localization of myosin-II isozymes in human cultured cells and blood cells. *J. Cell Sci.* 107:3077-3090

12. Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Kakano, T., Matsuura, Y. and Kaibuchi, K. (1996) Phosphorylation and activation of myosin by rho-associated kinase (rho kinase). *J. Biol. Chem.*, 271: 246-249.

13. Kimura, K, Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996) Regulation of myosin phosphatase by rho and rho-associated kinase. *Science* 273: 245-248.

14. Yamakita, Y., Yamashiro, S., and Mataumura, F. (1994) In vivo phosphorylation of regulatory light chain of myosinII suring mitosis of cultured cells. *J. Cell Biol.* 124:129-137.

15. Sanders, L.C., Matsumura, F., Bokoch, G.M., and de Lanerolle, P. (1999) Inhibition of myosin light chain kinase by p21-activated kinase. *Science* 283:2083-2085.

16. Edwards, D.C., Sanders, L.C., Bokoch, G.M. and Gill, G.N. (1999) Activation of LIM-kinase by PAK couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nature Cell Bio.* 1:253-259.

17. Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O. and Caroni, P. (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* 393:805-809.

18. Mira, J-P., Benard, V., Groffen, J., Sanders, L.C. and Knaus, U.G. (2000) Constitutive activation of Rac3 mediates proliferation in breast cancer cells by p21-activated kinase-dependent pathways. *PNAS* 97:185-189

19. Manser, E., Huang, H.-Y., Loo, T.-H., Chen, X.-Q., Dong, J.-M., Leung, T. and Lim, L. (1997) Expression of constititively active α-PAK reveal effects of the kinase on actin and focal complexes. *Mol. Cell. Biol.* 17:1129-1143.

20. Dharmawardhane, S., Sanders, L.C., Martin, S.S., Daniels, R.H. and Bokoch, G.M. (1997) Localization of p21 activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells. *J. Cell Biol.* 138:1265-1278.

21. Sells, M.A., Knaus, U.G., Ambrose, D., Bagrodia, S., Bokoch, G.M., and Chernoff, J. (1997) Human p21 activated kinase regulates actin reorganization in mammalian cells. *Curr. Biol.* 7:202-210

Appendices:

- PAK activity is important for cytoskeletal changes.
- PAK phosphorylates and inactivates MLCK.
- PAK phosphorylates and activates LIM-Kinase.
- PAK activity decreases MLC phosphorylation, in vivo.
- PAK is hyperactive in some breast cancer cell lines.
- Breast cancer cells with hyperactive PAK mislocalize PAK.
- Breast cancer cells with hyperactive PAK have an abnormal cytoskeletal morphology and a decrease migratory potential.

Publications

Sanders, L.C., Matsumura, F., Bokoch, G.M., and de Lanerolle, P. (1999) Inhibition of Myosin Light Chain Kinase by p21-Activated Kinase. *Science* 283:2083-2085.

Edwards, D.C., Sanders, L.C., Bokoch, G.M. and Gill, G.N. (1999) Activation of LIM-Kinase by PAK Couples Rac/Cdc42 GTPase Signalling to Actin Cytoskeletal Dynamics. *Nature Cell Bio.* 1:253-259

Mira, J-P., Benard, V., Groffen, J., Sanders, L.C. and Knaus, U.G. (2000) Constitutive Activation of Rac3 Mediates Proliferation in Breast Cancer Cells by p21-Activated Kinase-Dependent Pathways. *PNAS* 97:185-189

King, C.C., Sanders, L.C., and Bokoch, G.M. (2000) In Vivo Activity of Wild Type and Mutant PAKs. In *methods in Enzymology – Regulators and Effectors of Small GTPases.* Edited by W.E. Balch, C.J. Der and A. Hall. Published by Academic Press. In Press

Presentations

1999 Keynote Address, University of Calif., Riverside - Graduate Student Retreat
1999 Invited Speaker, American Society for Cell Biology Meetings, Washington D.C.